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Secreted acetylcholinesterase: non-classical aspects of a classical enzyme

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Recent evidence suggests that termination of cholinergic transmission is just one of the many ways in which acetylcholinesterase (AChE) could influence neuronal function. Neuronal AChE can be secreted from several brain regions, while purified AChE possesses several properties (in addition to its cholinesterase activity) that can affect neuronal function, including the abilities to influence certain membrane conductances, enhance excitatory amino acid transmission and hydrolyse peptides. Loss of AChE and its non-classical actions would have a profound effect on brain function in neurodegenerative diseases such as Alzheimer's disease where there is widespread loss of AChE-containing neurons.

Acetylcholinesterase (AChE) plays an established role in cholinergic transmission by hydrolysing, and thus terminating the action of, the transmitter acetylcholine (ACh). However, the classical idea that the presence of AChE is associated only with cholinergic transmission[1] is incorrect. High levels of AChE activity are found in non-neuronal tissues such as erythrocytes[2], platelets[3] and lymphocytes[4], where a role for AChE in cholinergic transmission is hard to envisage. Within the nervous system itself there is a wide disparity between the distributions of AChE and the more faithful cholinergic marker choline acetyltransferase (ChAT), the synthesizing enzyme for ACh (Refs 5, 6). In certain brain regions, such as the substantia nigra, cerebellum, globus pallidus and hypothalamus, there is a high concentration of AChE, while ChAT activity is disproportionately low and there are few cholinergic synapses. That is suggestive of a non-cholinergic distribution of AChE. Indeed, comparison of AChE and ChAT at the level of the single neuron demonstrates that many non-cholinergic neurons contain AChE (Ref. 7). Such findings suggest that AChE may possess functions other than the hydrolysis of ACh.

Such a non-cholinergic distribution of AChE is even more apparent when one considers the developing nervous system. Intense amounts of AChE activity appear transiently in many immature non-cholinergic neurons during development with no accompanying ChAT or ACh. Studies by several authors in diverse tissues and species[8–11] indicate that there may be a common pattern underlying this transient appearance of AChE: appearance of AChE coincides with the outgrowth and extension of long projecting neurites from these neurons but precedes the process of active synaptogenesis. Such findings have prompted speculation that during development AChE has a non-cholinergic function aiding neurite outgrowth.

Another surprising feature of AChE is its considerable structural polymorphism[12]. AChE exists in asymmetric forms, containing between four and twelve catalytic subunits attached to a membrane-anchored collagen tail, and globular forms (G1, G2 and G4) containing one, two or four catalytic subunits as membrane-bound or soluble entities. Alternative mRNA splicing may also result in AChE species with differing amino acid sequences[13]. Some of this heterogeneity is undoubtedly related to the different mechanisms by which AChE may be anchored to the membrane, but the presence of such a large number of molecular forms, including catalytically inactive forms[14], may be related to additional non-cholinergic functions of AChE.

Secretion of AChE

Further evidence suggesting that the classical view of AChE function is inadequate comes from studies demonstrating that AChE is secreted from neuronal structures in several areas of the brain, including the hippocampus[15–17], hypothalamus[18], striatum[19], substantia nigra[20] and cerebellum[21]. Indeed, examination of the amino acid sequence of AChE shows that it has the features of a secreted extracellular protein rather than a transmembrane one since it lacks long hydrophobic stretches, other than that forming the signal peptide[22].

Studies utilizing intrahippocampal push–pull cannulae show that secretion of AChE within the hippocampus probably originates from the terminals of the cholinergic septohippocampal pathway since hippocampal AChE secretion is enhanced by electrical stimulation of this pathway[17] and abolished by lesioning the pathway[16]. Furthermore, hippocampal AChE secretion appears to be associated with cholinergic transmission since it is evoked by stimulation of local muscarinic cholinergic receptors[15]. Within a cholinergic region such as the hippocampus one can envisage a cholinergic role for secreted AChE that fits these data. ACh, once released, would stimulate presynaptic autoreceptors resulting in negative feedback regulation of ACh secretion and at the same time evoking AChE secretion. The secreted AChE could then hydrolyse ACh within the region of the synaptic cleft more effectively than membrane-bound AChE since it is able to diffuse freely.

However, in other regions of the brain, AChE is secreted in a non-cholinergic capacity. Within the cerebellar cortex, levels of AChE are high, while there is very little ACh and ChAT (Ref. 5), and no convincing electrophysiological evidence for cholinergic transmission[23]. Studies *in vivo* using intracerebellar push–pull cannulae have demonstrated Ca^{2+}-dependent K^+-evoked secretion of AChE from the cerebellar cortex that is unaffected by stimulation of cholinergic receptors by local infusion of carbachol[21]. One of the principal sources of AChE within the cerebellar cortex is the climbing fibre system[5], which is probably aspartadergic, and projects from the

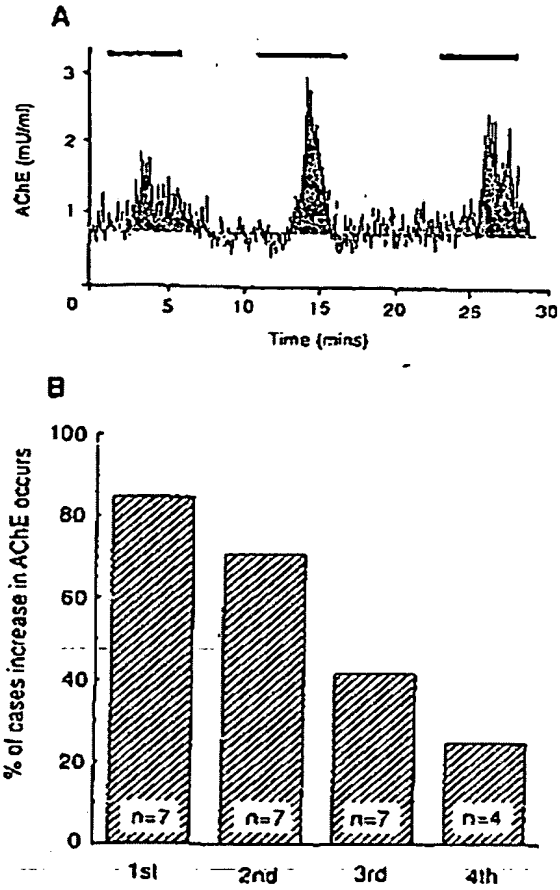

Fig. 1. *Release of AChE into nigral perfusates of awake guinea pigs as determined by an 'on-line' chemiluminescent reaction for AChE.* (A) *A typical trace of AChE release obtained during three five-minute periods of the guinea pig walking in a motorized tread wheel (shown by bars), separated by two five-minute stationary periods. Release of AChE occurs in a pulsatile fashion and is composed of 'peaks' of AChE (shaded areas). Peaks of protein release occur most often while the animal is moving. However, the duration of the peak of AChE activity is always much shorter than that of the associated period of locomotor activity.* (B) *During repeated bouts of activity, the likelihood of the corresponding peaks of protein release occurring decreases. During the first episode of activity, six out of the seven animals exhibit an increase in AChE release, but by the fourth episode only one out of four animals produce a corresponding increase.* (Reproduced, with permission, from Ref. 28.)

inferior olive onto Purkinje cells. This pathway can be stimulated by systemic application of harmaline, and this produces a marked increase in the release of AChE within the cerebellar cortex[21], suggesting that secreted AChE originates from this pathway and is hence associated with excitatory amino acid transmission.

Similarly in the substantia nigra, studies using push–pull cannulae and 6-hydroxydopamine lesions have demonstrated that AChE is secreted from the dendrites of non-cholinergic, dopaminergic neurons[5]. Despite a sparse cholinergic input to these neurons[20], the release of AChE is unaffected by local application of various cholinergic agonists[24,25] and is therefore unrelated to cholinergic transmission. Dopaminergic drugs and 5-hydroxytryptamine (5-HT) do affect secretion of AChE within the nigra, but in a manner unrelated to their effect on the firing rate of nigral neurons[20]. This observation has led to the proposal that dendritic AChE release is related to the presence of a high-threshold Ca^{2+} spike ($HTSg_{Ca}$) in the nigrostriatal dendrites. This is supported by the demonstration in intracellular recording studies that 5-HT (Ref. 20) and amphetamine[26], both of which evoke nigral AChE secretion, also enhance the $HTSg_{Ca}$. However, cholinergic agonists also enhance the $HTSg_{Ca}$ (Ref. 26), but they have no effect on nigral AChE secretion[24].

Several recent studies have used an 'on-line' chemiluminescent assay for monitoring AChE activity of push–pull perfusates, allowing secretion to be observed on a timescale more comparable with that of physiological events (Fig. 1). Such studies have shown that in the awake animal, release of AChE from the nigra is pulsatile in nature[27] and is frequently, but not always, associated with periods of locomotion, both voluntary[28] and induced by a rotating wheel[28]. Stimulation of sensory inputs by light flashes also enhances nigral AChE secretion, implying that this phenomenon may be important in visuo–motor interactions[29].

Such demonstrations that AChE is secreted from non-cholinergic neurons have prompted suggestions that AChE could perform a non-classical function within the extracellular space and so influence the behaviour of surrounding neurons.

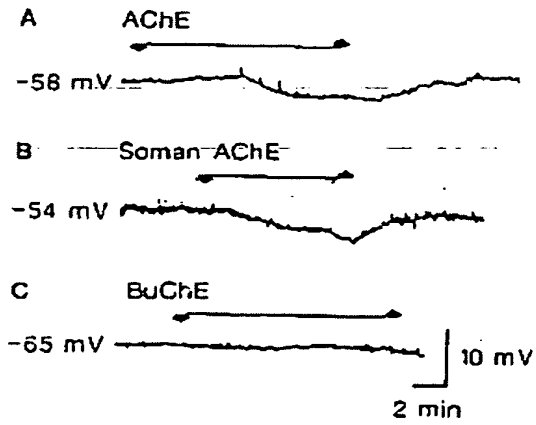

Fig. 2. *Typical effects of various cholinesterase preparations pressure-ejected in the vicinity of the distal dendrites of nigral cells.* (A) *Application of AChE (Sigma electric eel, type V–S) at an approximate rate of 2.7 mU per minute for the period denoted by the line leads to a reversible hyperpolarization of approximately 4 mV.* (B) *AChE, pre-treated with an irreversible inhibitor of its cholinesterase activity (Soman), leads to a hyperpolarization comparable with that seen in* (A) (C) *Butyrylcholinesterase (BuChE), of concentration in the electrode ten times that of* (A) *and* (B) *and applied for a comparable period of time, has no effect on membrane potential* (Reproduced, with permission, from Ref. 32.)

Non-classical actions of AChE

The first indications that secreted AChE could influence the behaviour of surrounding neurons arose from demonstrations by Greenfield[5] and colleagues that microinfusions of purified AChE into the nigra *in situ* depressed both the spontaneous and stimulus-linked firing of nigral neurons, and also induced locomotor activity[20], stereotypy[20] and chewing movements[29]. Such actions could not be attributed to hydrolysis of endogenous ACh since they were not evoked by butyrylcholinesterase (BuChE), which is also cholinolytic. More recently such non-classical actions of AChE have been confirmed at the cellular level in both the substantia nigra and the cerebellum using intracellular recordings in guinea pig brain slices.

In the substantia nigra, bath application[10,11] or pressure ejection of AChE in the region of the dendrites[32] induces a reversible hyperpolarization of cells (Fig. 2), with an accompanying decrease in input resistance. These effects of AChE have been attributed to its opening of K^+ channels since they were unaffected by blockade of Na^+ or Ca^{2+} channels, and had a reversal potential near the K^+ equilibrium potential[32]. Recent experiments suggest that the actual site of action of AChE on nigral cells is on an ATP-sensitive K^+ channel since its effects can be blocked by the sulphonylurea tolbutamide, and are enhanced in low glucose concentrations[41]. Despite the sparse cholinergic projections to these neurons, these effects of AChE are not due to termination of cholinergic transmission as they are not blocked by pretreatment of the AChE with the irreversible cholinesterase inhibitor Soman, nor are they mimicked by BuChE (Refs 30–32). Indeed, an enzymatic action can probably be ruled out completely since boiled AChE can still produce such effects[41]. However the precise mechanism by which AChE could influence membrane channels has still to be found.

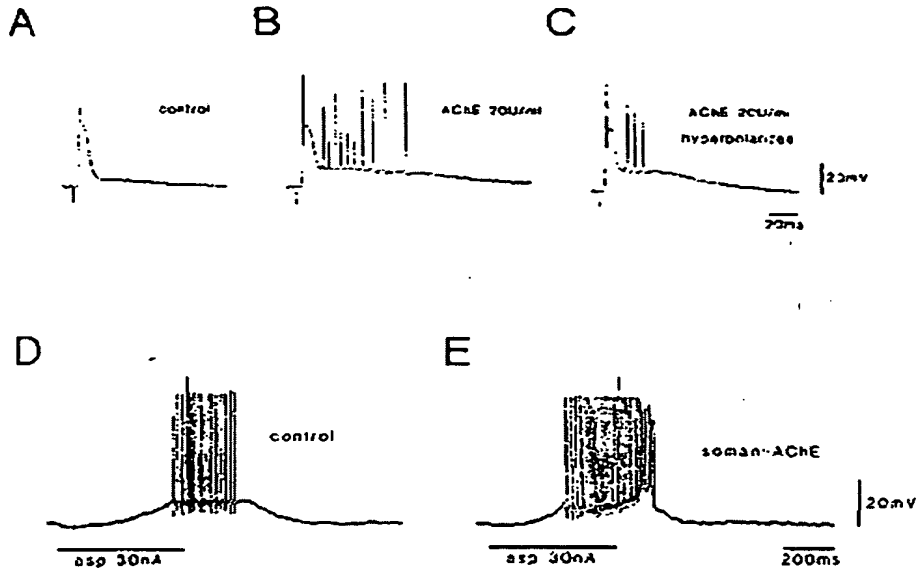

Fig. 3. *Typical effects of AChE preparations on the responses of cerebellar Purkinje cells. Typical all-or-nothing synaptic responses evoked in cerebellar Purkinje cells by stimulation of their climbing fibre input obtained in control solution (A) and in the presence of AChE (B), (C). Addition of AChE produced an enhancement of the late plateau-like part of the climbing fibre response (CFR). The effect was so large in this particular cell that the Purkinje cell fired Na^+ spikes during the late part of the CFR (A), (B). Even when the cell was hyperpolarized by 4 mV by injecting inward current through the recording electrode, the cell generated Na^+ spikes after AChE treatment (C). (D), (E) Typical effect of AChE on the response of cerebellar Purkinje cells to an excitatory amino acid. These particular traces show the response to iontophoresis of aspartate recorded before (D) and 20 minutes after Soman-treated AChE was introduced into the recording chamber (E). Although the esterase activity was completely eliminated by Soman, the effect produced was comparable to that of untreated AChE: responses to aspartate became larger and faster after AChE. Hence the effects of AChE are not due to hydrolysis of ACh.* (Adapted, with permission, from Ref. 23.)

These actions of AChE are probably selective for a subpopulation of dopaminergic neurons situated in the rostral portion of the substantia nigra[31]. Such cells are capable of generating low-threshold Ca^{2+} spikes ($LTSg_{Ca}$) that in turn generate a burst of action potentials, but only when they are rapidly depolarized from an initially hyperpolarized state[20]. Hence, as proposed by Greenfield[20], AChE, by hyperpolarizing such cells, would enable a subsequent excitatory input t the cell to generate a $LTSg_{Ca}$ and burst-firing. Such an action is consistent with the view that AChE is a modulator that enhances the sensitivity of nigral cells to synaptic inputs.

Intracellular recording studies performed by Appleyard and Jahnsen[23] in the cerebellar cortex (Fig. 3) also support this view of AChE as a modulator of neuronal function, although in this case a different mechanism is involved. Bath application of AChE produces a considerable enhancement of the responses of cerebellar Purkinje cells to iontophoretic applications of the excitatory amino acids aspartate and glutamate. Synaptic responses evoked by stimulation of climbing fibres are also enhanced by AChE, particularly a late plateau-like part of the response. These particular effects of AChE are probably due to a blockade of excitatory amino acid reuptake, leading to enhanced levels of the released transmitter, since they are abolished in the presence of the reuptake blockers D,L-2-amino-4-phosphonobutyric acid and dihydrokainate.

Autoradiographic studies indicate that there are high levels of sulphonylurea binding sites in the cerebellar cortex, particularly in the molecular layer that contains the dendrites of Purkinje cells[33]. This suggests that ATP-sensitive K^+ channels are abundant in this region. Indeed single-channel recordings have demonstrated the presence of such channels in cultured cerebellar neurons[34], although these were probably granule cells rather than Purkinje cells. However, AChE does not appear to promote opening of K^+ channels in Purkinje cells since no significant effect on either the membrane potential or input resistance has been observed[23], despite the cells being held at potentials well away from E_K. However, in Purkinje cells, AChE does appear to affect another membrane conductance; it reduces a non-inactivating

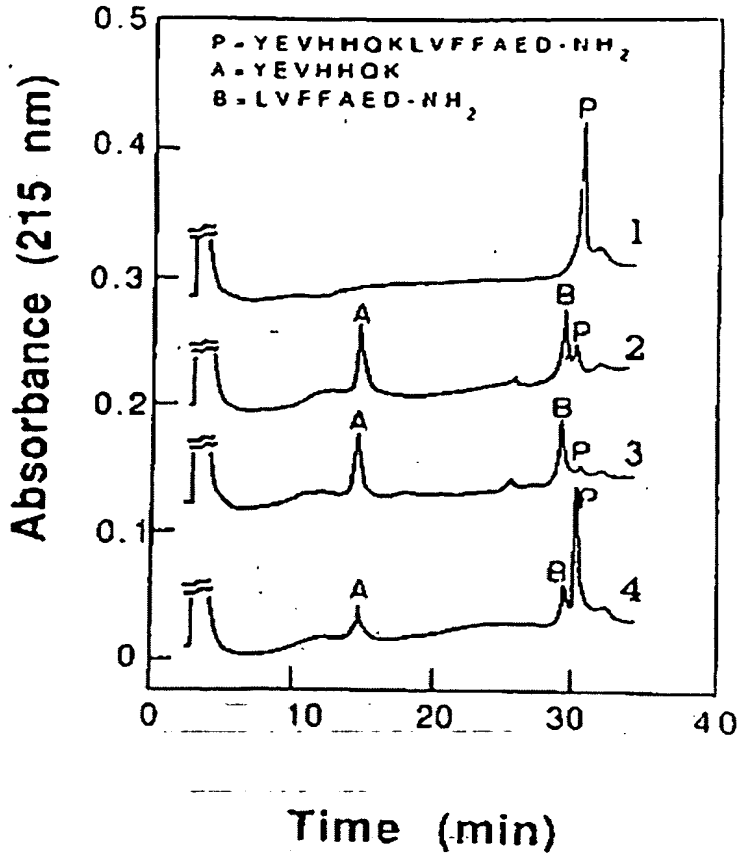

Fig. 4. Demonstration of protease activity associated with different preparations of affinity-purified AChE. Each trace shows a reversed-phase high-pressure liquid chromatogram of an AChE-associated protease (AChE-AP) digest of a synthetic peptide corresponding to a fragment of the amyloid precursor protein ($\beta A4_{10-23}$) on a Novapak C18 column (0.4 × 150 mm). The synthetic peptide (P) was incubated without protease (incubation 1), or in the presence of bovine calf serum AChE-AP (incubation 2), or electric eel AChE-AP (incubation 3) for 1 h. In incubation 4, the peptide was incubated with 400 000 fold purified human brain AChE for 24 h. Control experiments confirmed that the peptide was completely stable over 24 h in the absence of any added protease. In each case AChE-AP cleaved the synthetic peptide producing two smaller fragments A and B, the identities of which were confirmed by amino acid analysis and by comparison of the elution times of the same two peptides generated by digestion with trypsin. (Reproduced, with permission, from Ref. 36.)

Na^+ current, thereby altering the firing pattern of Purkinje cells in response to prolonged depolarization[25].

None of these effects of AChE on cerebellar Purkinje cells can be attributed to an effect on cholinergic transmission, since there is no evidence for such transmission in the first place, nor can the effects be blocked by the irreversible cholinesterase inhibitor Soman, nor mimicked by BuChE (Ref. 23).

Biochemical studies, by Small and colleagues, on purified AChE from several different sources, including (fetal calf serum[35], eel electroplax[35] and human brain[36], have demonstrated that a tryptic-like protease activity (Fig. 4) and a Ca^{2+}-stimulated exopeptidase activity is associated with AChE. The association of such activities with AChE has raised the possibility that AChEs are proteases, or zymogens of proteases, that are secreted into the extracellular environment where they could perform a number of roles, including peptide hydrolysis[37] and developmental roles, such as regulation of cellular proliferation and neurite outgrowth[35]. Such a function would explain the transient appearance of AChE in many developing neurons[9–11].

However, there is currently disagreement about whether such activities are due to the presence of minor contaminants[34–40] or if they are truly intrinsic to AChE[35,36]. While there is certainly evidence for the presence of contaminating bovine trypsin (used in the preparation method) in crude commercial sources of electric eel AChE (Ref. 40), the presence of tryptic activity in other sources of AChE, such as that obtained from human brain, is less easy to explain away, since no trypsin was used in their preparation[36], although the possibility of other contaminating endogenous proteases must be borne in mind. Furthermore, Small[38] asserts that the tryptic activity associated with serum AChE and with highly purified preparations of electric eel AChE is not inhibited by soybean trypsin inhibitor (unlike the activity associated with cruder preparations of electric eel AChE (Ref. 39)), and is therefore not due to the presence of trypsin. Small[35,37] also asserts that the tryptic-like protease activity associated with highly-purified preparations of AChE cannot be removed by affinity chromatography using either specific substrates for AChE or proteases or by antibodies to AChE, although separation can be achieved from crude preparations of electric eel AChE. However, Araki *et al.*[39] believe that physical separation of the AChE and protease activities can be achieved, since they were able to remove 99.985% of the protease activity from commercial electric eel preparations using a three-stage chromatographic procedure. Indeed, physical separation of the active sites of the protease and AChE activities is indicated by the finding that the protease activity is not affected by reversible inhibitors of AChE, such as BW284c51 or edrophonium[38,38,39], despite its shared sensitivity to organophosphates.

Small[35] has proposed that the tryptic activity of AChE is due to the presence of a 25 kDa polypeptide that arises by autolytic cleavage of one 70 kDa subunit (in an analogous manner to trypsinogen) and remains tightly bound to the other three subunits of the G4 form (and the remaining 50 kDa fragment), enabling co-purification and, in certain circumstances, separation of the two enzymatic activities. Opponents of this theory would point out that trypsin itself is a 25 kDa protein, although it is worth noting that Araki *et al.* have confirmed the presence of 70, 50 and 25 kDa species in their highly purified preparation of electric eel AChE (Ref. 39). In support of his theory Small points out that C-terminal sequences of AChE from *Torpedo* and *Drosophila*, determined by molecular cloning techniques, exhibit a degree of sequence identity with serine proteases. In particular, a region of *Drosophila* AChE between residues 579 and 614 possesses about 40% sequence similarity to the active site region of porcine trypsin[34], although it remains to be seen whether *Drosophila* AChE also

exhibits tryptic activity. No doubt this controversy will continue until a purified source of recombinant AChE has been assessed for intrinsic peptidase activity.

The presence of tryptic-like activity in preparations of AChE, whether intrinsic or as contaminants, also raises the question of whether the effects of AChE on nigral and cerebellar cells can be attributed to protease action. It would seem to be unlikely since more highly purified sources of electric eel AChE, presumably with less contaminating bovine trypsin, had a greater effect on nigral cells than the crude commercial preparation, although strangely more actual AChE activity (and presumably, therefore, more AChE protein) of the cruder preparation was required[32]; the effects of AChE on both nigral[30] and cerebellar cells[23] persisted after treatment with Soman, which will inhibit serine proteases such as trypsin; and in the nigra the effects could still be produced by boiled preparations of AChE that presumably had no remaining enzymatic activity associated with them (although this was not confirmed by a protease assay)[32].

It can therefore be concluded that AChE does indeed possess a number of associated non-cholinergic actions that can have diverse effects on the activity of neurons when it is secreted from a neighbouring neuronal structure. However, in many cases the exact mechanism by which these effects are produced is unclear, and the search for these will undoubtedly form the basis of future studies. Ideally, such studies will use a well-characterized source of purified recombinant mammalian AChE so that artefacts due to the presence of minor contaminants can be eliminated.

Clinical Implications

It should be obvious from the above discussion that any disease in which alterations of levels of secreted AChE are a prime feature will result in abnormal neuronal functioning, arising not only from the classical effects on cholinergic transmission but also from the non-cholinergic effects on membrane channels, excitatory amino acid transmission and peptides. Two neurodegenerative diseases in particular, Parkinson's disease and Alzheimer's disease (AD), are associated with loss of AChE in various brain regions, and the implications of non-classical functions of AChE for each of these diseases will be considered.

In the movement disorder Parkinson's disease there is a marked loss of the dopaminergic nigrostriatal neurons that secrete AChE from their dendritic regions in the nigra and their terminals in the striatum[20]. Although nothing is presently known about the role of this secreted AChE in the striatum, we have seen that in the nigra, secretion of AChE is related to locomotor activity[27,29], and serves to enhance the responsiveness of surrounding nigral neurons to subsequent activation of synaptic inputs. This may be exhibited behaviourly as facilitation of movement, as application of exogenous AChE to the nigra of awake animals evokes motor activity such as circling[20], stereotypy[20] and chewing[20]. We can speculate that in Parkinson's disease not only are there fewer nigral neurons but, due to loss of their secreted AChE, the remaining neurons will differ in their response to synaptic inputs. This situation will serve to exacerbate any locomotor defects associated with cell loss.

In AD there is a widespread and marked loss of AChE in both cholinergic and non-cholinergic regions of the brain that exhibit the typical neuropathological abnormalities (neuritic plaques and neurofibrillary

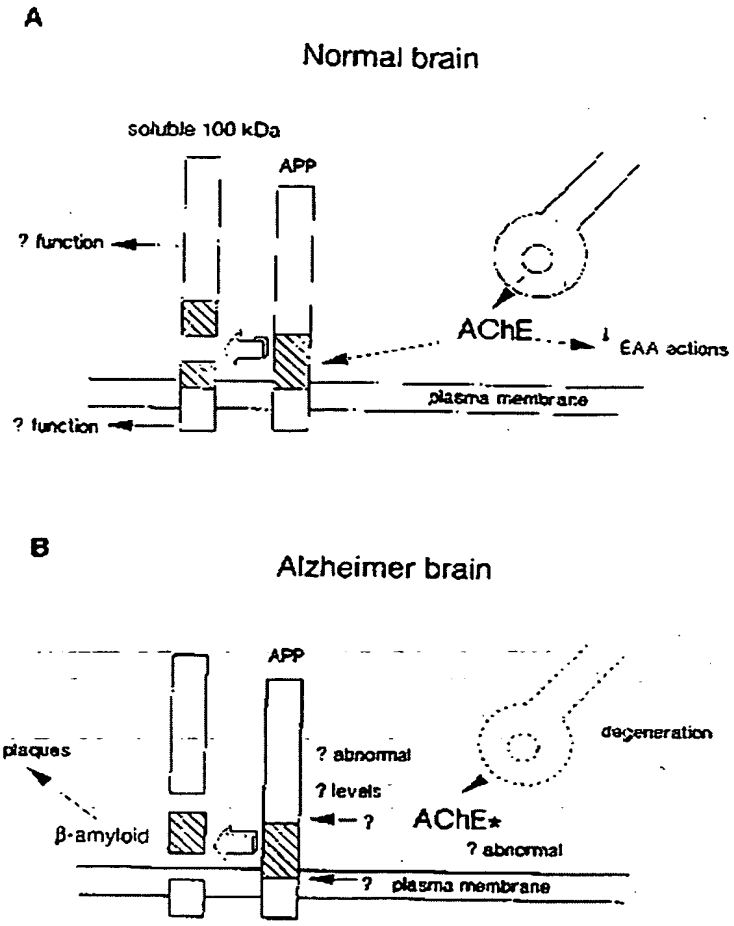

Fig. 5. *Schematic diagram showing a hypothetical relationship between AChE loss and plaque deposition in the brains of Alzheimer's patients.* **(A)** *In the normal brain, AChE is secreted from neuronal structures and has a number of non-classical actions on surrounding neurons, including potentiation of excitatory amino acid (EAA) transmission. A tryptic-like activity associated with the secreted AChE is capable of cleaving the transmembrane protein APP at an extracellular site within the region corresponding to the β-amyloid sequence. This reaction produces a soluble extracellular fragment of 100 kDa that could then affect surrounding neurons by its ability to act as a protein inhibitor, and also produces a membrane/cytoplasmic fragment of unknown function. At the same time the β-amyloid portion of APP is disrupted.* **(B)** *In the brains of Alzheimer's patients there is a widespread degeneration of neurons containing AChE, resulting in a decreased level of secretion. The form of AChE that is secreted in AD may also be abnormal. Both these phenomena will reduce the non-classical effects of AChE on surrounding neurons. APP will no longer be cleaved by AChE, so allowing abnormal processing, possibly by lysosomal enzymes, liberating the β-amyloid fragment that can then be deposited in plaques. Any function associated with the normal products of APP cleavage will be severely disrupted. In genetic forms of AD, the situation would be exacerbated by the presence of abnormal forms or elevated levels of APP.*

tangles) associated with the disease. In addition, the remaining AChE may represent an abnormal form of the enzyme as it exhibits a lower pH optimum compared to forms present in age-matched control human brains[42], and it is less sensitive to substrate inhibition[43]. Secreted AChE appears to be similarly affected by the disease since markedly lower levels of AChE are present in ventricular cerebrospinal fluid of patients[41,44], presumably due to decreased secretion from forebrain regions affected by the disease. Secreted AChE also appears to be abnormal in AD patients since it is not subject to substrate inhibition[41], and an abnormal band is present in cerebrospinal fluid subjected to isoelectric focusing[45]. An abnormal structure of the enzyme could have drastic effects on its ability to perform the non-classical functions described above, with dire consequences for the activity of the surrounding neurons.

If AChE has non-classical actions in brain regions affected by AD, then lack of normal secreted AChE would diminish the activation of ATP-sensitive K^+ channels and reduce the blockade of non-inactivating Na^+ channels. This would tend to lead to increased excitability of neurons and increased incidence of spontaneous firing. At the same time the neurons would become less responsive to their synaptic inputs, particularly those utilizing an excitatory amino acid transmitter or those capable of evoking a LTS_{Ca}. In short, there would be a severe disruption of normal neuronal function.

Any protease activity associated with AChE would also be severely reduced in AD patients, and such an effect may explain an apparent link between AChE and plaque formation[46,47] (Fig. 5). Senile plaques often have a dense protein core composed of β-amyloid (βA4), which is derived from abnormal cleavage of a larger precursor protein (APP) present as a transmembrane protein of unknown function in normal brains[48]. In normal brains, APP undergoes proteolytic cleavage at another cleavage site (in the middle of the βA4 sequence) located in the extracellular portion of the molecule, generating a soluble extracellular fragment, identified as the protease inhibitor protease nexin 2, and a membrane/cytoplasmic fragment of unknown function. This reaction also prevents generation of βA4 (Ref. 48). Small *et al.* have shown that purified AChE from a number of different sources, including normal human brain, is capable of producing normal cleavage of APP (Ref. 36). Hence it is tempting to allocate a role for secreted AChE in the normal processing of APP. In AD brains with lowered levels of normal AChE there would be less processing of APP, allowing abnormal cleavages to produce βA4 that is then deposited in plaques, perhaps together with the abnormal AChE, thus explaining its presence in the plaques[47]. Any function of the normal products of APP cleavage would also be adversely affected.

It can be seen from this discussion that, regardless of the exact mechanism, loss of secreted AChE in a disease state, or indeed elevated levels, would have profound effects on neuronal function with far-ranging consequences for associated behaviours. Future research into the mechanisms of non-classical actions of AChE could provide vital clues for the treatment, prevention or both of debilitating conditions such as AD and Parkinson's disease.

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